sponded to areas of degenerate or vacuolated ER. These structures were absent from AcchiA- or uninfected cells. Finally, SF9 cells infected with AcMNPyV and stained with the same reagent produced very low levels of fluorescence (Figure 2c).

The data presented in this paper show that removing the chiA gene from an AcMNPyV recombinant expressing the nAChRz gene reduces accumulation of the protein product within the ER. It remains to be seen if this reduction in intracellular accumulation of nAChRz is accompanied by an increase in localization of the protein in the plasma membrane. Given that baculoviruses have evolved to maximize production of occlusion bodies in virus-infected insect larvae, it is likely that many other genes could be removed from the genome without affecting replication in cell culture, but possibly with positive benefits for recombinant protein production.


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Molecular chaperones improve functional expression of the serotonin (5-hydroxytryptamine) transporter in insect cells

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The serotonin (5-hydroxytryptamine) transporter (SERT) belongs to a small family of neurotransmitter transporters [1–3] with substrates that include neurotransmitters (serotonin, noradrenaline, dopamine, γ-aminobutyric acid, glycine) and other small organic molecules (creatine, proline, betaine, taurine). Uptake is driven by the influx of Na+ and Cl− down their concentration gradients; intracellular K+ or H+ ions are also required for transport by some of these transporters. The cDNA sequences of these transporters predict a family of proteins with a number of common characteristics, i.e. 12 putative transmembrane domains, with the N- and C-term in the cytoplasm, and a large extracellular loop between transmembrane domains 3 and 4 that is always N-glycosylated. The sites of potential N-glycosylation and their number is not conserved across the family, with the number of potential N-glycans varying between 1 and 4. It has also been proposed that there is a disulphide bond within this extracellular loop between two Cys residues that are absolutely conserved across the family [4,5].

SERT is a particularly interesting neurotransmitter transporter because it is the binding site for anti-depressant drugs and it is also inhibited by cocaine and amphetamines [6,7]. Site-directed-mutagenesis data have delineated particular residues that are important for drug

Abbreviations used: SERT, serotonin (5-hydroxytryptamine) transporter; p.i., post-infection; RT155, 3β-carboxymethoxy-3β-(4-iodophenyl)tropane; ER, endoplasmic reticulum; BiP, immunoglobulin heavy chain-binding protein; CXN, calnexin; CRT, calreticulin; dNM, 1-deoxynojirimycin; SERT-QQ, unglycosylated SERT.

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very strong polyhedrin promoter that is turned on about 20 h post infection (p.i.). Other weaker translational recombinant membrane protein are usually polyhedrin gene [19,20]. The recombinant viruses active SERT from them [11-15]. Therefore, SERT has been overexpressed in a variety of systems, the most successful being in insect cells using the baculovirus expression system [16-18].

The baculovirus expression system relies on the production of recombinant baculoviruses with the cDNA of interest replacing the non-essential polyhedrin gene [19,20]. The recombinant viruses are used to infect insect cells, usually Sf9, SF21 or Hi5 cell lines, to produce the recombinant protein. Usually the cDNA is placed downstream of the very strong polyhedrin promoter that is turned on about 20 h post infection (p.i.). Other weaker promoters can also be used, such as the immediate early promoter ie1 that is turned on immediately the virus enters the cell, and the basic protein promoter that is turned on between 12 and 24 h p.i. Cells infected with baculovirus will eventually die 4-5 days after infection. The baculovirus expression system has been used to express a wide variety of integral membrane proteins in a functional form [21,22]. However, the levels of functional recombinant membrane protein are usually 10-100 times lower than obtained for soluble proteins. In addition to functional protein, there are often considerable quantities of inactive membrane protein [21].

SERT was expressed in Sf9 insect cells [16] from the polyhedrin promoter in a recombinant baculovirus (bvSERT). bvSERT-infected cells were capable of [3H]serotonin transport and specifically binding the SERT inhibitor 185I-RTI55 [2β-carbomethoxy-3 β-(4-iodophenyl)tropane]. The $K_d$ for inhibitor binding (0.22 nM) was identical to that of SERT in native tissues, although the $K_m$ for serotonin uptake (78 nM) was slightly lower than expected. Western-blot analysis of membranes prepared from bvSERT-infected cells showed three different forms of SERT: unglycosylated SERT (54 kDa) was the predominant species, with a smaller amount of N-glycosylated SERT (60 kDa) and an SDS-resistant aggregate (130 kDa). Two lines of evidence suggest that the unglycosylated SERT is predominantly inactive. First, expression of SERT in SF21, Hi5 and MG1 insect cells resulted in the same level of functional expression, but the amount of unglycosylated SERT seen in Western blots was about 100-times higher in the Hi5 and MG1 cells than in SF21 cells [16]. Secondly, solubilization of SERT selectively extracts the glycosylated form with no loss of active SERT (C. G. Tate, unpublished work). N-glycosylation is not essential for the function of SERT per se. Mutation of the two Asn residues that are normally N-glycosylated in SERT resulted in an unglycosylated transporter (SERT-QQ) that still bound 185I-RTI55 and transported [3H]serotonin with unchanged $K_a$ and $K_m$ values [16]. However, the amount of SERT-QQ was decreased 20-fold compared with the native transporter. This suggested that N-glycosylation is probably important for the folding or stability of SERT.

One of the major problems associated with using baculovirus for expressing SERT is the relatively low levels of functional transporter obtained. Expression levels of SERT expressed on a small scale in unagitated tissue-culture flasks were about (0.4-0.5)×10⁶ copies per cell (7–9 pmol/mg of membrane protein) [16]. A contributing factor to the low expression levels in insect cells could be the baculovirus-mediated cell death which is accelerated on expression of SERT, resulting in impaired protein synthesis and membrane insertion earlier than expected [17]. Certainly, functional SERT is only produced up to 48 h p.i.; any further expression resulted in unglycosylated, inactive SERT [16]. The accelerated cell death on SERT expression is unlikely to be via apoptosis, because co-expression of the apoptosis inhibitor Bcl2 did not delay the phenomenon [22], despite the ability of Bcl2 to inhibit cell death by wild-type baculovirus [23]. It is also unlikely that SERT-induced cell death is due to the activity of SERT itself, because inclusion of SERT inhibitors (imipramine, RTI55 or cocaine) in the cell medium during expression had no effect ([17] and C. G. Tate, unpublished work). Lowering the temperature of the cell culture, either on addition of virus or 24 h p.i. also did not increase the expression of functional SERT (C. G. Tate, unpublished work).

Another strategy that improves protein expression in Escherichia coli and yeast is fusing a highly expressed polypeptide or protein at the N-terminus of the membrane protein [21,22]. Fusion of small epitope tags to the N- or C-termini of SERT did not affect functional expression [16]. Fusion of glutathione S-transferase to the N-
terminus of SERT [17] resulted in a 3-fold decrease in functional expression levels to 2.5 pmol/mg of membrane protein. Currently, the only strategy that has improved SERT expression is the co-expression of molecular chaperones [18].

Molecular chaperones are a diverse collection of proteins that interact transiently with an unfolded or misfolded protein, thus assisting it to fold correctly [25,26]. A variety of integral membrane proteins, including transporters [27–30], ion channels [31,32] and G-protein-coupled receptors [33–35], are known to interact in vivo with molecular chaperones. It seemed reasonable to think that if molecular chaperones that were normally required for the folding of SERT in native tissues were limiting in insect cells, this could lead to misfolded SERT; co-expression of these molecular chaperones with SERT could therefore improve the amount of functional SERT expressed. Unfortunately, it was not known which molecular chaperones were required for the normal folding of SERT. Therefore, a number of endoplasmic reticulum (ER)-resident molecular chaperones that were known to interact with other membrane proteins were co-expressed with MycSERT (MycSERT contained an N-terminal calmodulin-binding domain and a c-Myc epitope, and a C-terminal His$_{16}$ tag [18]) to see if they could improve its expression. Indeed, if the co-expressed molecular chaperone did increase the functional expression of SERT in insect cells, then the molecular chaperone was a potential candidate for assisting the folding of SERT in native tissues.

The molecular chaperones initially used included the immunoglobulin heavy chain-binding protein (BiP), calnexin (CXN) and calreticulin (CRT). In addition, a number of ER-resident foldases were co-expressed with MycSERT, which included protein disulphide isomerase, peptidyl-prolyl cis-trans isomerase (PPI), NinA (a membrane-bound PPI from Drosophila) and ERp57 (an N-glycan specific protein disulphide isomerase). Only CXN gave a large increase in the level of functional MycSERT expressed in insect cells; on day 3 p.i. CXN improved functional MycSERT expression by 3-fold compared with the control [18]. BiP improved the expression of MycSERT by 1.4-fold and CRT by 1.3-fold [18].

### Figure 1

**Western blot of CXN expressed in insect cells from three different promoters**

A culture of SF9 cells (10$^6$ cells/ml) in a shaker flask was infected with a multiplicity of infection (MOI) 5 of recombinant baculoviruses expressing CXN from either the ie1 promoter, the basic protein (bp) promoter or the polyhedrin (ph) promoter. The control virus bv-pVL does not express any protein from the polyhedrin promoter. The baculovirus bvMycSERT was added at an MOI of 5 to all the cultures. Cells were grown shaking at 150 rev/min at 27 °C for 3 days. Cells (10 ml) were removed at 1, 2 and 3 days p.i. and 1 ml of membranes were prepared [17], separated by SDS-PAGE and Western blotted. The blot was probed with an anti-CXN antibody (StressGen). Numbers below each lane refer to the day p.i. when the cells were harvested. The two panels were part of the same blot and were probed and developed simultaneously. The bands representing CXN are indicated by arrows.
A slight effect was also seen with PPI, but none of the other foldases or molecular chaperones improved functional MycSERT expression (C. G. Tate, unpublished work). Co-expression of MycSERT and CXN with either BiP, CRT or ERP57 consistently led to a decrease in functional MycSERT expression in comparison with MycSERT co-expressed with just CXN; this may be a reflection of trying to simultaneously express three membrane/secreted proteins together rather than on their failure to interact with MycSERT [18].

Alternative baculovirus promoters were used in an attempt to improve the level of CXN expressed and to enhance SERT activity (Figure 1). CXN was expressed from the basic protein promoter to about the same levels as from the polyhedrin promoter, and the levels of MycSERT expression were increased on day 2 p.i. by the same amount (Figure 2). The weak Iel promoter produced only a modest increase in CXN compared with the level seen in insect cells infected with the control baculovirus bv-pVL, and no increase in MycSERT activity was observed.

The interaction between MycSERT and the molecular chaperones was further studied by co-immunoprecipitation [18]. Immunoprecipitation of MycSERT from solubilized membranes using an anti-c-Myc monoclonal antibody resulted in the co-immunoprecipitation of CXN and CRT, suggesting a direct interaction between MycSERT, CXN and CRT. This was confirmed by expression experiments in the presence of 1-deoxynojirmycin (dNJM) to inhibit the SERT–chaperone interaction. Both CXN and CRT interact with proteins via the N-glycan trimming intermediate GlcNac\textsubscript{2}Man\textsubscript{2}Glc [36–39], following the removal of two glucose residues by dNJM-inhibitable glucosidases I and II. Inclusion of dNJM in the cell medium prevented co-expressed CXN from increasing the functional expression of MycSERT [18]. In addition, CRT and CXN could not be co-immunoprecipitated with MycSERT if dNJM was included in the growth medium. Thus it seems likely that the effects of CXN and CRT on increasing the functional expression of MycSERT are due to a direct interaction between MycSERT and the molecular chaperones.

One prediction from the fact that dNJM abolished the interaction between MycSERT and CXN was that the functional expression of an unglycosylated SERT mutant would not be increased by CXN co-expression. However, co-expression of CXN with SERT-QQ led to a slight, but reproducible, improvement in SERT-QQ expression [18]. This suggested that other interactions between CXN and SERT-QQ were present apart from the CXN–N-glycan interaction.

One unexplained result was that dNJM did not decrease the level of functional MycSERT expression in the absence of co-expressed CXN [18]. There was clearly a protein in insect cells that cross-reacted with the anti-CXN antibody that was identical in size to mammalian CXN (Figure 1), but the inability of dNJM to decrease functional MycSERT expression suggested that it was not involved in folding MycSERT. Either insect CXN does not interact with mammalian MycSERT or it was in a different membrane to the site of MycSERT synthesis. The possibility that insect CXN was not inhibited by dNJM also cannot be excluded.

All the co-expression experiments described above used MycSERT, which contained an N-terminal calmodulin-binding domain and a C-terminal His\textsubscript{10} tag for purification, in addition to the c-Myc epitope. The tags were not expected to interact directly with the ER-resident molecular chaperones because the N- and C-termini of SERT are thought to reside in the cytoplasm. However, co-expression of CXN with untagged SERT led to a smaller increase in functional expression than was seen for tagged SERT (1.4-fold and 3-fold respectively) [18]. This suggested that the tags were actually having a subtle effect on either the folding pathway or kinetics of folding of
MycSERT, which resulted in greater interaction with co-expressed molecular chaperones.

Co-expression of CXN with other integral membrane proteins may also improve their levels of functional expression. It would be expected that multi-subunit N-glycosylated ion channels may in particular benefit from this approach, because of the need to maintain individual subunits post-translationally in an unaggregated state suitable for interaction with their partners.

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